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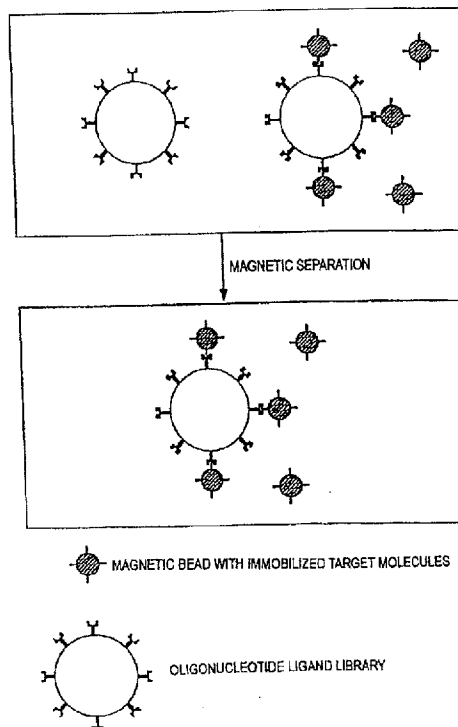
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(54) Title: METHOD OF ISOLATING TARGET SPECIFIC OLIGONUCLEOTIDE LIGANDS

(57) Abstract

This invention relates to methods of preparing oligonucleotide libraries, isolating oligonucleotide aptamers to target molecules from the libraries, and using the aptamers to purify target molecules by affinity separation. Specific oligonucleotide aptamers to erythropoietin were isolated accordingly.



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DESCRIPTION

Method Of Isolating Target Specific Oligonucleotide Ligands

Related Applications

This application is based on provisional U.S. Ser. No. 5 60/082,405, filed April 20, 1998, the contents of which, including drawings and sequence, are hereby incorporated by reference into the present application.

Field Of The Invention

10 The present invention relates to the synthesis and identification of oligonucleotide ligands of target molecules and uses thereof. The invention also relates to Fluorescence Activated Cell Sorter analysis, i.e., FACScan flow cytometry, and other convenient separation means.

Background Of The Invention

15 SELEX, i.e., Systematic Evolution of Ligands by Exponential Enrichment, is a process of isolating oligonucleotide ligands of a chosen target molecule (see Tuerk and Gold, Science 249:505-510, (1990), U.S. Patents 5,475,096, 5,595,877, and 5,660,985). SELEX as described in 20 Tuerk and Gold involves admixing the target molecule with a pool of oligonucleotides (e.g., RNA) of diverse sequences; retaining complexes formed between the target and oligonucleotides; recovering the oligonucleotides bound to the target; reverse-transcribing the RNA into DNA; 25 amplifying the DNA with polymerase chain reactions (PCR); transcribing the amplified DNA into RNA; and repeating the cycle with ever increasing binding stringency. Three enzymatic reactions are required for each cycle. It usually takes many cycles (e.g., between 12-15 cycles) to isolate 30 aptamers of high affinity and specificity to the target. An aptamer is an oligonucleotide that is capable of binding to

an intended target substance but not other molecules under the same conditions.

Bock et al., (1990) Nature 355:564-566, describes another approach of isolating aptamers. Bock's process is different from that of Tuerk and Gold in that only one enzymatic reaction is required for each cycle (i.e., PCR) because the nucleic acid library in Bock's method is comprised of DNA instead of RNA. The identification and isolation of aptamers of high specificity and affinity with the method of Bock et al. still requires repeated cycles in a chromatographic column.

Conrad et al., (1996) Methods in Enzymol. 267:336-367, describes a variety of methods for isolating aptamers, all of which employ repeated cycles to enrich target-bound ligands and require a large amount of purified target molecules.

Summary Of The Invention

The present invention features a simple, speedy and cost efficient method of identifying aptamers to specific target molecules. It allows for the identification and isolation of aptamers without numerous cycles of selection and amplification.

Within the scope of this invention, Applicant successfully identified and isolated synthetic single-stranded oligonucleotides of high affinity to a target molecule of interest, erythropoietin. In addition, Applicant successfully used a synthetic single-stranded oligonucleotide in an affinity matrix to purify another molecule of interest, prothrombin. The Applicant's success in these endeavors suggests a broad-spectrum applicability of the invention that is not limited to the specific biologic molecules studied herein. Those of skill in the art will appreciate that such can be applied to any molecule or compound of interest, biologic or otherwise, that has an

affinity for oligonucleotide ligand sequences of the type described herein.

In a first aspect, this invention features a method that makes use of magnetic separation to identify an aptamer
5 which specifically binds to a target molecule of interest by magnetic separation. In this method, the target molecule is conjugated to a magnetic substance. After allowing the target molecule to mix with a collection of candidate oligonucleotides under conditions suitable for complex
10 formation, i.e., binding of target molecule with aptamer(s), magnetic force is applied to separate aptamer(s) from the rest of the candidate oligonucleotides that have little or no affinity for the target molecule. Preferably, this method contains the following steps:

- 15 a) providing a collection of candidate oligonucleotides attached to a plurality of solid supports, wherein candidate oligonucleotides of different nucleotide sequences are attached to different solid supports and all candidate oligonucleotides attached to a single solid
20 support are of the same nucleotide sequence;
- b) providing a plurality of magnetic beads covered with the target molecule;
- c) admixing the collection of candidate oligonucleotides on solid supports with the magnetic beads
25 covered with the target molecule under conditions suitable for the binding of oligonucleotide ligands to the target molecule, wherein magnetic bead conjugates complex with one or more solid supports bearing aptamers to the target molecule of interest;
- 30 d) applying magnetic force to isolate one or more solid supports attached with magnetic beads; and
- e) identifying oligonucleotides present on the isolated solid supports.

A collection of candidate oligonucleotides is generated
35 using conventional synthesis techniques. Preferably, each oligonucleotide in the collection contains both a randomized

sequence as well as at least one adjacent primer sequence for amplification and/or sequencing. Candidate oligonucleotides include single-stranded and double-stranded RNA or DNA of any length. A candidate oligonucleotide may
5 contain modified or derivatized groups known in the art, especially those identified in U.S. Patents 5,582,981 and 5,660,985, such as analogous forms of purines and pyrimidines and analogous forms of ribose and deoxyribose.

A target molecule can be any molecule capable of
10 forming a complex with an oligonucleotide, including, but not limited to, peptides, proteins, enzymes, antibodies, hormones, glycoproteins, polymers, polysaccharides, nucleic acids, small organic compounds such as drugs, dyes, metabolites, cofactors, transition state analogs and toxins.

15 Specific target molecules of interest include molecules of biological and physiological relevance in both prokaryotic and eukaryotic organisms, particularly mammals. Examples of such biologically significant molecules in mammals include, but are not limited to, erythropoietin,
20 tissue plasminogen activator, granular colony stimulating factor (G-CSF), growth hormone (GH), endostatin (O'Reilly et al., (1997) Cell 88: 277-285), interferons, interleukins, chemokines (Shi et al., (1997) FASEB J. 11:1330; Bubrovsky et al., (1996) PNAS, USA 92: 700-709), enzymes such as SOD
25 (Yoshikai et al., (1995) Cancer Res. 55(8) 1617-1620) and amylase, antibodies (particularly the constant "Fc" regions thereof), OKT3 (Ho et al., (1998) Science 280: 1866-1867), serum proteins (e.g., Factor VIII (Papadopoulos-Eleopoulos et al., (1990) Genetica 95: 35-50), Factor VIX, plasminogen,
30 antithrombin III (Jones et al., (1992) Br. J. Cancer 66: 744-747), albumin, protein C (Griffin et al., (1993) Blood 82: 1989-93), etc.), and vaccines (e.g., HbsAg (Davis et al., (1994) Vaccine 12: 1503-1509), etc.). The physiological significance of most of these, and many other molecules, may
35 similarly be found in Goodman and Gilman's The Pharmacological Basis of Therapeutics, 8th ed., (1990)

Pergamon Press, Elmsford, New York. Those of skill in the art will appreciate that a virtually unlimited number of other target molecules may also be used with the claimed methods.

- 5 The solid support can be anything suitable for attaching oligonucleotides, including, but not limited to, resin beads, controlled pore glass (e.g., Maskos and Southern, (1992) Nucleic Acid Res. 20:1679-1684), polystyrene (e.g., McCollum and Andrus, (1991) Tetrahedron
10 Lett. 32:4069-4072), PEG-polystyrene (e.g., Gao et al., (1991) Tetrahedron Lett. 32:5477-5480), Teflon (e.g., Arnold, US Patent 5,362,866), cellulose (e.g., Crea and Horn, (1980) Nucleic Acid Res. 8:2331-2348). In a preferred embodiment, the oligonucleotides of diverse sequences are
15 synthesized on the solid supports (e.g., Pon, (1993) Methods Mol. Biol. 20:465-496). In another preferred embodiment, the solid support is of a size observable under an optical microscope. Such a size facilitates the selection of individual solid supports following magnetic separation.
- 20 Oligonucleotides attached to individual solid supports can be sequenced directly to obtain the identity, consensus sequence and/or structure information of the aptamers. Preferably, the solid support has a diameter of about 50 μm to about 500 μm , more preferably about 70 μm to about 150
25 μm , and even more preferably about 90 μm to about 120 μm . The target molecule is optionally labeled with a fluorescent label to facilitate the identification and selection of solid supports which form complexes with the target molecule through aptamer/target molecule interaction.
- 30 The magnetic beads covered with the target molecule are preferably much smaller than the solid supports attached with oligonucleotides. For example, the magnetic beads may have a diameter of about 1 μm to about 10 μm , or about 2 μm to about 5 μm .
- 35 If too many solid supports are selected by the above described process, one can increase the binding stringency

and isolate a more limited set of solid supports attached with oligonucleotides of higher affinity to the target molecule. If none or too few solid supports are selected by the process, one can lower the binding stringency to isolate
5 more solid supports. The phenomenon of stringency is known by those of skill in the art to be routinely affected and modulated by parameters such as temperature and ion strength of solution.

In a second aspect, this invention features a method of
10 identifying an aptamer which specifically binds to a target molecule by fluorescence activated sorting. In this method, the target molecule contains or is attached with a fluorescent label. After allowing the target molecule to mix with a collection of candidate oligonucleotides under
15 conditions suitable for aptamer binding and complexing, fluorescence activated sorting is applied to separate aptamer(s) complexed with the fluorescent target molecule from the rest of the candidate oligonucleotides. Preferably, this method contains the following steps:

- 20 a) providing a collection of candidate oligonucleotides attached to a plurality of solid supports, wherein candidate oligonucleotides of different nucleotide sequences are attached to different solid supports, each support having identical oligonucleotide sequences bound
25 thereto;
- b) providing fluorescence labeled target molecules;
- c) admixing the candidate oligonucleotides on solid supports with the target molecules under conditions suitable for oligonucleotide/target molecule binding;
- 30 d) isolating one or more solid supports attached with the fluorescence labeled target molecules; and
- e) identifying oligonucleotides attached to the isolated solid supports.

In a preferred embodiment, the solid supports attached
35 with the fluorescence labeled target molecules are isolated by fluorescence activated sorting. Because FACscan can sort

particles into different fractions according to their fluorescence intensity, this method allows a series of oligonucleotides to be sorted according to relative affinity strength to target molecule, i.e., solid supports bearing
5 higher affinity oligonucleotides attract more fluorescence labeled target molecules.

In another preferred embodiment, the fluorescence labeled target molecules are linked to magnetic beads and magnetic separation is applied before the fluorescence
10 activated sorting. This step increases the speed of FACScan by reducing the number of particles to be sorted.

In a third aspect, this invention features a method of identifying an aptamer which specifically binds to a target molecule. This method contains the following steps:

15 a) providing a collection of oligonucleotides of diverse sequences in a liquid, wherein each of said oligonucleotides is linked with a detectable label such as a fluorescent label;

20 b) attaching a plurality of solid supports, e.g., resin beads, to target molecules, said target molecules exposed on the support surfaces and accessible to oligonucleotides;

25 c) admixing the collection of oligonucleotides with the solid supports covered with the target molecule under conditions suitable for oligonucleotide binding to the target molecule;

d) selecting solid supports linked to fluorescence labeled oligonucleotides by fluorescence activated sorting; and

30 e) identifying the oligonucleotides linked to the selected solid supports, e.g., by amplification and sequencing.

By utilizing magnetic separation and/or fluorescence activated sorting, the processes of this invention allow the
35 isolation and identification of an aptamer in a single step without repeated cycles. The processes described above for

isolating aptamers may be used in lieu of or in combination with conventional methods in the art, including, but not limited to, those described in U.S. Patents 5,475,096 and 5,582,981.

5 Following the identification of aptamers with the processes described in this application, one can further delineate consensus secondary structures and primary sequences, tertiary structures, motifs, and activity boundaries using methods known in the art, including, but
10 not limited to, those described in U.S. patent 5,595,877. Nucleic acid ligands, once identified, can be further modified or derivatized using methods known in the art, including, but not limited to, those described in U.S. patent 5,660,985.

15 In an example, oligonucleotides that specifically bind to erythropoietin were isolated and identified with a method of this invention. Specifically, the present invention provides single stranded DNA ligands to erythropoietin according to the method described above. The sequences of
20 these ligands are listed in Figure 4. Also included is consensus DNA sequence identified by sequence homology and binding ability to erythropoietin. Thus, in another aspect, this invention features an oligonucleotide aptamer binding to erythropoietin with an affinity of no less than 10^{-4} M.
25 The affinity ranges from 10^{-3} to 10^{-8} M, preferably 10^{-6} to 10^{-8} M.

 In a preferred embodiment, the oligonucleotide aptamer is substantially homologous, preferably no less than 70%, more preferably no less than 80%, and even more preferably
30 no less than 90%, to the sequences of Figure 4. Homology is defined as identical bases at the same position within the range of sequence nucleotide positions that is compared. Preferably, the homology is from about 75% to about 100% within the 8 base consensus region in Figure 4, Group I. In
35 another preferred embodiment, such an oligonucleotide

aptamer to erythropoietin contains a sequence as shown in FIG 4.

Oligonucleotides designed to closely resemble or match the consensus sequence would have high affinity for the target molecule. For example, an oligonucleotide containing multiple consensus sequences separated by spacers would have high affinity for the target molecule. Specifically, an oligonucleotide containing two consensus regions linked by a 6 base spacer binds to erythropoietin as strong as F15.

Other features and advantages of the invention will be apparent from the following drawings and detailed description of the invention and from the claims.

Brief Description Of The Drawings

Figure 1 is a schematic diagram showing a method of identifying oligonucleotide aptamers that uses magnetic separation.

Figure 2 is a schematic diagram showing a method of identifying oligonucleotide aptamers that uses fluorescence activated sorting.

Figure 3 is a schematic diagram showing another method of identifying oligonucleotide aptamers that uses fluorescence activated sorting.

Figure 4 is a diagram showing the sequences of aptamers to erythropoietin which are isolated from an oligonucleotide library. A consensus sequence is identified in the aptamers of Group I. Group II is comprised of oligonucleotides not containing the consensus sequence.

Figure 5 is an autoradiogram showing the specific binding of an isolated oligonucleotide, F15 (Seq ID No: 8), to erythropoietin in native polyacrylamide gel electrophoresis. It also shows that oligonucleotide F15 does not bind to the negative control, i.e., an equal amount of bovine serum albumin.

Figure 6 comprises two radiograms verifying the purification of prothrombin with an oligonucleotide ligand matrix.

(A): Collected fractions on 7.5% SDS-PAGE of. Lane 1, crude human plasma; lane 2, molecular weight marker (BioRad, low range); lane 3, wash fraction; lane 4, eluted prothrombin fraction.

(B): Western blot analysis of collected fractions. Molecular weight and prothrombin positions are marked by arrows.

Detailed Description Of The Invention

1. Selecting aptamers by magnetic bead separation

Figure 1 illustrates a process of selecting aptamers by magnetic separation. In this method, an oligonucleotide library is synthesized on TentaGel beads (polyethylene glycol grafted polystyrene-divinylbenzene polymer beads), each covered with a plurality of unique and identical oligonucleotides. The target molecule is linked (conjugated) to magnetic beads (Streptavidine-coated Dynal spheres M280). The diameter of resin beads for oligonucleotides is substantially larger than that of the magnetic beads (from about 10 to about 100 times larger). For example, the resin beads may have a diameter of about 90 μm and are observable under an optical microscope, while the magnetic beads have a diameter of about 2.8 μm .

The oligonucleotide library is brought into contact with the target molecule at a concentration preferably below the desired affinity. For example, if affinity of 10^{-6} M or higher is desired, the concentration of the target molecule is preferably below 10^{-6} M. The selection stringency can be adjusted by choosing the appropriate binding and washing conditions, and picking oligonucleotide beads above a defined fluorescence intensity. The selection stringency can be manipulated with salt concentrations between 50 mM and 250 mM NaCl, preferably between 100 mM and 200 mM NaCl.

After allowing the oligonucleotide library to interact with the target molecule-magnetic conjugate, magnetic force is applied to the mixture to separate complexed oligonucleotide resin beads from uncomplexed resin beads. The mixture in a microfuge tube is subject to a magnetic field and magnetic beads are retained on the side of the tube while non-magnetic beads can be washed away with buffers. The washing procedure is repeated several times and more stringent conditions can be applied to select for high affinity beads. The retained beads are examined, e.g., using fluorescence microscopy when the target molecules are fluor- labeled. Individual oligonucleotide resin beads can then be singled out and subject to sequencing and/or amplification.

2. Selecting aptamers using flow cytometry (1)

Figure 2 illustrates a process of selecting aptamers by fluorescence-activated sorting. In this method, an oligonucleotide library is synthesized on resin beads. The target molecule is conjugated with a fluorescent label.

The oligonucleotide library is brought into contact with the target molecule under conditions suitable for binding. The selection stringency can be adjusted by choosing the appropriate binding and washing conditions.

After allowing the oligonucleotide library to interact with the target molecule, the mixture is supplied to a FACS machine to select oligonucleotide resin beads bearing fluorescent label. Oligonucleotide resin beads of varying fluorescence intensity can be separated automatically by programming the FACS machine to collect beads of specific fluorescence intensity in individual sample containers.

In a preferred embodiment, the target molecule:ligand complex is therefore marked and identified using both a fluorescent label and a magnetic label. After allowing the oligonucleotide library to interact with the target molecule, oligonucleotide aptamers for the target molecule

are selected by magnetic force and sorted using fluorescence activated sorting.

3. Selecting aptamers by flow cytometry (2)

Figure 3 illustrates a second process of selecting
5 aptamers by fluorescence activated sorting. In this method,
a soluble oligonucleotide library is synthesized and each
oligonucleotide is marked with a fluorescent label. The
target molecule is linked to a solid support such as a bead.

The oligonucleotide library is brought into contact
10 with the target molecule under conditions suitable for
binding. The selection stringency can be adjusted by
choosing the appropriate binding and washing conditions.

After allowing the oligonucleotide library to interact
with the target molecule, the mixture is supplied to a FACS
15 machine to select target molecule beads covered with
fluorescent label.

4. Preparing a library of oligonucleotide ligands

An oligonucleotide in a candidate library of this
invention contains a variable region flanked by one or two
20 constant regions. The variable region contains randomized
sequences while the constant region(s) contains primer
binding sites for amplification and/or sequencing of the
oligonucleotide aptamer, and/or restriction sites for
cloning. The variable region is designed to contain
25 molecular diversity from which specific ligands can be
selected.

In a preferred embodiment, an oligonucleotide library
is prepared on solid support such as PEG resin beads. Each
oligonucleotide is covalently linked to the resin. The
30 molecular diversity is generated by split-pool strategy
commonly practiced in combinatorial chemistry processes
(Gallo et al., (1994) J. Medicinal Chem. 37:1233-1251).
Oligonucleotides on each individual bead are preferably
unique and identical. The oligonucleotide library therefore
35 comprises a plurality of beads each representing a unique
oligonucleotide construct. The size of a library is

correlated to the complexity of oligonucleotide ligands. For example, 4^{13} (i.e., 67 million) individual beads are required for a 13 mer library which is completely randomized and representative. 4^{15} (i.e., 1 billion) beads are required
5 for a 15 mer library which is completely randomized and representative.

The oligonucleotide can be DNA, RNA, their derivatives, mimetics, or a combination thereof.

In another preferred embodiment, the oligonucleotide or
10 the solid support thereof is tagged with a marker, e.g., a fluorescent label which allows selection, e.g., by fluorescence activated sorting (FACS).

In yet another preferred embodiment, a soluble oligonucleotide library is synthesized with each
15 oligonucleotide marked with a fluorescent label.

5. Preparing the target molecule for the selection process

A target molecule can be linked to a solid support such as a magnetic bead, either directly by covalent bonding or
20 indirectly through a linker or precoated molecule such as a strepavidin coated bead. For example, erythropoietin molecules can be biotinylated with NHS-biotin and linked to strepavidin coated magnetic beads.

Alternatively, the target molecule can be labeled with
25 a fluorescent probe such as fluorescein to allow selection by fluorescence activated sorting (FACS).

6. Amplifying and sequencing the selected ligands

The selected oligonucleotides can be amplified with
polymerase chain reactions utilizing primers directed to the
30 constant regions of the oligonucleotides. The PCR products can be cloned and sequenced subsequently. Preferably, individual beads are sequenced directly with labeled primers, either manually or automatically.

Utility

The ligands isolated by the method of this invention can be used as affinity ligands to separate and purify target molecules, as probes to trace, monitor, detect and
5 quantitate target molecules, or to block, activate or catalyze reactions that are physiologically relevant to achieve therapeutic effect (see review by Gold (1995) J. Biol. Chem. 270:13581-4).

Oligonucleotide ligands so isolated have utilities
10 similar to antibodies. They can act as a pharmaceutical agent. They can bind to a specific target and direct specific molecules to a desired site, and/or they can inhibit or promote a physiologically relevant reaction to achieve a desired therapeutic effect. Various *in vivo*, *ex*
15 *vivo*, and *in vitro* methods can employ the principles taught herein, as will be appreciated by one of skill in the art.

Within respect to *in vitro* procedures, the applicant has found that isolated oligonucleotide aptamers can be used in affinity purification matrixes to purify target
20 molecules. Oligonucleotide aptamers are ideal for chromatographic separations of target molecules from contaminants. In a preferred embodiment, applicant has found that oligonucleotide aptamers are particularly useful for purifying target protein molecules from cell cultures or
25 cell extracts.

Specifically, applicant has demonstrated a one step purification of prothrombin from crude plasma. The immediate application of this technology is to purify
30 antibodies, enzymes, hormones, receptors, and factors that are used in research, development, diagnostic, pharmaceutical, industry applications.

Oligonucleotides of specific chiral properties can be used to separate chiral compounds and obtain optically pure chemicals.

35 Oligonucleotide ligands can also be used in place of antibodies in various research, development and diagnostic

applications such as blotting techniques, flow cytometry, immunoassays, strip assays, immunohistological techniques, affinity sensors, etc. Oligonucleotide can further be used to monitor, trace, detect and quantitative desired target
5 such as proteins, antibodies, microbes, virus, bacteria, macromolecules, and small molecules. Oligonucleotide ligands will also be valuable tools for proteomics studies of protein and their function.

10 Example 1. Isolating Oligonucleotide Ligands for Erythropoietin

Synthesizing a Soluble Oligonucleotide Library

Deoxyoligonucleotides each containing 40 bases of random sequence flanked by two 18 base primer binding sites and a 5' fluorescein label were synthesized using standard
15 techniques (Genosys). The 76 mer library has the following formula:

5'-F-label-CATGA ACTAG TGGAT CCG -40nt- TTGGT ACCCA ATTCG CCC-3' (seq ID NO: 1).

The 5' end constant region is comprised of 5'-CATGA
20 ACTAG TGGAT CCG-3' (seq ID NO: 2), while the 3' end constant region is comprised of 5'-GGGCG AATTG GGTAC CAA-3' (seq ID NO: 3).

Restriction sites BamHI and Kpn I are designed into the constant regions to facilitate cloning and the production of
25 probes.

Immobilizing EPO to Streptavidin Beads

Purified erythropoietin in 100 mM sodium bicarbonate buffer (pH 8.0) was biotinylated with NHS-biotin (molar ratio biotin:target 35:1, Calbiochem) for four hours at room
30 temperature. Free biotin was removed by dialyzing against phosphate buffered saline. Biotinylated erythropoietin was linked to streptavidin beads (M280, Dynal) by incubating at 4°C overnight.

Isolating Erythropoietin Binding Oligonucleotide Aptamers

The fluorescein tagged oligonucleotide library (119 nmol, contains 7×10^{16} molecular diversity) was dissolved in PBS and incubated with erythropoietin immobilized on Dynal beads at room temperature for 15 minutes. The beads were washed with PBS five times and subject to fluorescence activated cell sorting (Becton-Dickson, FACStar Plus). Beads with the highest fluorescent intensity (0.1% of the total 600,000 beads scanned) were collected in PBS and subject to polymerase chain reaction (PCR) with Taq polymerase (Life Technologies). A total of twenty-five to thirty cycles were performed on a Perkin-Elmer DNA Thermal cycler (denaturing at 94°C for 45 sec, annealing at 60°C for 45 sec, and chain extension at 72°C for 1 minute). The PCR products were verified on 2% agarose TBE electrophoresis and digested with restriction enzymes Kpn 1 and BamH1 (New England Biolabs). The digested fragments were ligated and cloned into vector pBlueScript KS (Stratagene) and transformed into bacterial host DH5 α . Individual colonies were identified.

Testing the Affinity of an Aptamer to Erythropoietin

Plasmid DNA was prepared using boiling miniprep procedures according to Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989). Plasmids were digested with Kpn I and BamHI, and the fragments were end-labeled using ^{32}P -ATP and kinase. After the restriction digestion, the sense strand contains protruding ends 8 bases longer than the antisense strand. The labeled fragment were then denatured by sequencing stop buffer and heated at 100°C for 2 min, and electrophoretically separated on a 15% urea-TBE polyacrylamide gel.

Single stranded radioactive ^{32}P labeled probes were excised from the gel and eluted in 0.5 M NaAc, 20 mM Tris, pH 7.5, 10 mM EDTA, 0.1% SDS at 65°C for 30 min. The probes

were then extracted with phenol, precipitated with tRNA and ethanol, and air dried.

The probes were dissolved in PBS. Binding assays were performed in PBS at room temperature for 15 min. The formed
5 complexes were electrophoretically resolved in a native 0.5x TBE 4% polyacrylamide gel, and then exposed on X-ray film (Kodak).

Erythropoietin-bound aptamers were identified thereby and sequenced using the following primers:

10 Sense primer (Seq ID NO: 4) 5'-AGCGGATAACAATTTACACAGGA-3'

Antisense primer (Seq ID NO: 5) 5'-CGCCAGGGTTTTCCAGTCACGAC-3'

Manual sequencing was performed according to the
15 manufacturer's instructions (Amersham USB).

A consensus sequence emerged from five sequenced aptamers as shown in Figure 4, Group I. This consensus sequence was found to bind erythropoietin, albeit with slightly lower affinity than the five aptamers identified..
20 Three other aptamers were found that shared no homology with the consensus sequence (Figure 4, Group II).

The minimal consensus sequence and constructs bearing several copies of consensus demonstrated more or less binding. The spaced region between the two consensus
25 sequences was found to affect binding ability. It was thus determined that oligonucleotides with more or less affinity for the target molecule of interest could be prepared and identified based on the consensus sequence.

The specificity of oligonucleotide ligand to EPO is
30 demonstrated in FIG 2 by its ability to bind to EPO and not an equi-molar amount of bovine serum albumin.

Example 2. Purification of Prothrombin with an Oligonucleotide Ligand Matrix

Oligonucleotide ligand against thrombin has been
35 isolated by Bock et al., Nature 355:584-566, 1992. The

converse utility of an oligonucleotide ligand as affinity matrix to purify protein from crude extracts is demonstrated by the following.

Biotinylated oligonucleotide ligand against thrombin is produced according to known procedures (e.g., Genosys). The sequence (Seq ID NO: 6) of the ligand is as follows:

5'-GGTTGGTGTG GTTGGATGAT GGTGGTGTG GTTGGTAAGT-3'-biotin

The ligand (99 nmol) is labeled with T4 kinase with ^{32}P -ATP (New England Biolabs). Free radioactivity is removed by passage through a sephadex G25 spin column. The labeled ligands were incubated with 0.5 ml streptavidin-agarose (Life Technologies) at 4°C overnight. Coupling efficiency is monitored by reduced radioactivity in the solution before and after incubation. Coupling efficiency was found to be 71%.

The matrix bound ligand is packed in a small column (BioRad) and washed with phosphate buffered saline. Human plasma (Calbiochem, 10 ml) is loaded on the column. After washing with PBS, the bound prothrombin is eluted with 1 M NaCl, 5 mM EDTA, 25 mM Tris, pH 7.4.

The collected sample is analyzed by 7.5% SDS-PAGE, shown in FIG 3A and identity of prothrombin verified by western blotting as shown in FIG 3B.

Alternatively, the ligand can be synthesized directly on a solid support, preferably polyethylene glycol grafted polystyrene beads or cellulose based membrane.

Example 3. Synthesis of Oligonucleotide Library on Solid Support

PEG-grafted polystyrene beads (TentaGel S) are purchased from Fluka. Loading is controlled by reacting the beads with defined amount of DMT-Cl and capping the remaining OH groups on the support. The oligonucleotides are synthesized on an ABI 394 DNA synthesizer using standard procedures. One gram of solid support is divided into four equal parts and synthesis performed in four columns. After

the primer synthesis, the supports are mixed together and divided into four equal parts again and an additional base synthesized. The mix-split process is repeated for synthesis of a total of 20 bases, each column having a
5 defined base for each of the 20 rounds. After the mix-split, the second primer is synthesized.

Synthesis is monitored by trityl efficiency and the polymerase chain reaction.

10 All publications noted, none of which is admitted prior art, are incorporated herein by reference, including nucleic acid and amino acid sequence listings.

One of skill in the art will appreciate that other aspects and embodiments are possible within the true scope
15 and spirit of the invention. The claims below are further illustrative.

Claims

1. A method for identifying an aptamer which specifically binds to a target molecule, comprising the steps of:

- 5 a) providing a collection of oligonucleotides attached to a plurality of solid supports, wherein oligonucleotides of different nucleotide sequences are attached to different solid supports and all oligonucleotides attached to a single solid support are of
10 the same nucleotide sequence;
- b) providing a plurality of magnetic beads having said target molecule immobilized on their surface;
- c) admixing said collection of oligonucleotides with said plurality of magnetic beads under conditions
15 suitable for the binding of oligonucleotide ligands to the target molecule;
- d) applying magnetic force to isolate one or more solid supports attached with magnetic beads; and
- e) identifying oligonucleotides attached to said
20 one or more isolated solid supports.

2. The method of claim 1, wherein said solid supports are resin beads and said step d) comprises selecting resin beads attached with magnetic beads.

3. The method of claim 2, wherein said resin beads
25 have a diameter of about 50 um to about 500 um.

4. The method of claim 2, wherein said resin beads have a diameter of about 70 um to about 150 um.

5. The method of claim 2, wherein said resin beads have a diameter of about 90 um to about 120 um.

6. The method of claim 1, wherein said magnetic beads have a diameter of about 1 μm to about 10 μm .

7. The method of claim 1, wherein said magnetic beads have a diameter of about 2 μm to about 5 μm .

5 8. The method of claim 1, wherein said target molecule is labeled with a fluorescent probe.

9. The method of claim 1, wherein said collection of oligonucleotides are synthesized on said plurality of solid supports.

10 10. A method for identifying an aptamer which specifically binds to a target molecule, comprising the steps of:

15 a) providing a collection of oligonucleotides attached to a plurality of solid supports, wherein oligonucleotides of different nucleotide sequences are attached to different solid supports and all oligonucleotides attached to a single solid supports are of the same nucleotide sequence;

20 b) providing a plurality of said target molecules labeled with fluorescent probe;

 c) admixing said collection of oligonucleotides with said plurality of said target molecules under conditions suitable for the binding of oligonucleotide ligands to the target molecule;

25 d) isolating one or more solid supports attached with fluorescent probe; and

 e) identifying oligonucleotides attached to said one or more isolated solid supports.

11. The method of claim 10, wherein said fluorescence 30 labeled target molecules are linked to magnetic beads.

12. The method of claim 11, further comprising the step of applying magnetic force to isolate solid supports attached with magnetic beads after step c) and before step d).

5 13. A method for identifying an aptamer which specifically binds to a target molecule, comprising the steps of:

- 10 a) providing a collection of fluorescence labeled oligonucleotides of different nucleotide sequences;
- b) providing a plurality of solid supports having said target molecule immobilized on their surface;
- c) admixing said collection of oligonucleotides with said plurality of solid supports under conditions suitable for the binding of oligonucleotide ligands to the
- 15 target molecule;
- d) isolating solid supports attached with fluorescence labeled oligonucleotides; and
- e) identifying oligonucleotides attached to said isolated solid supports.

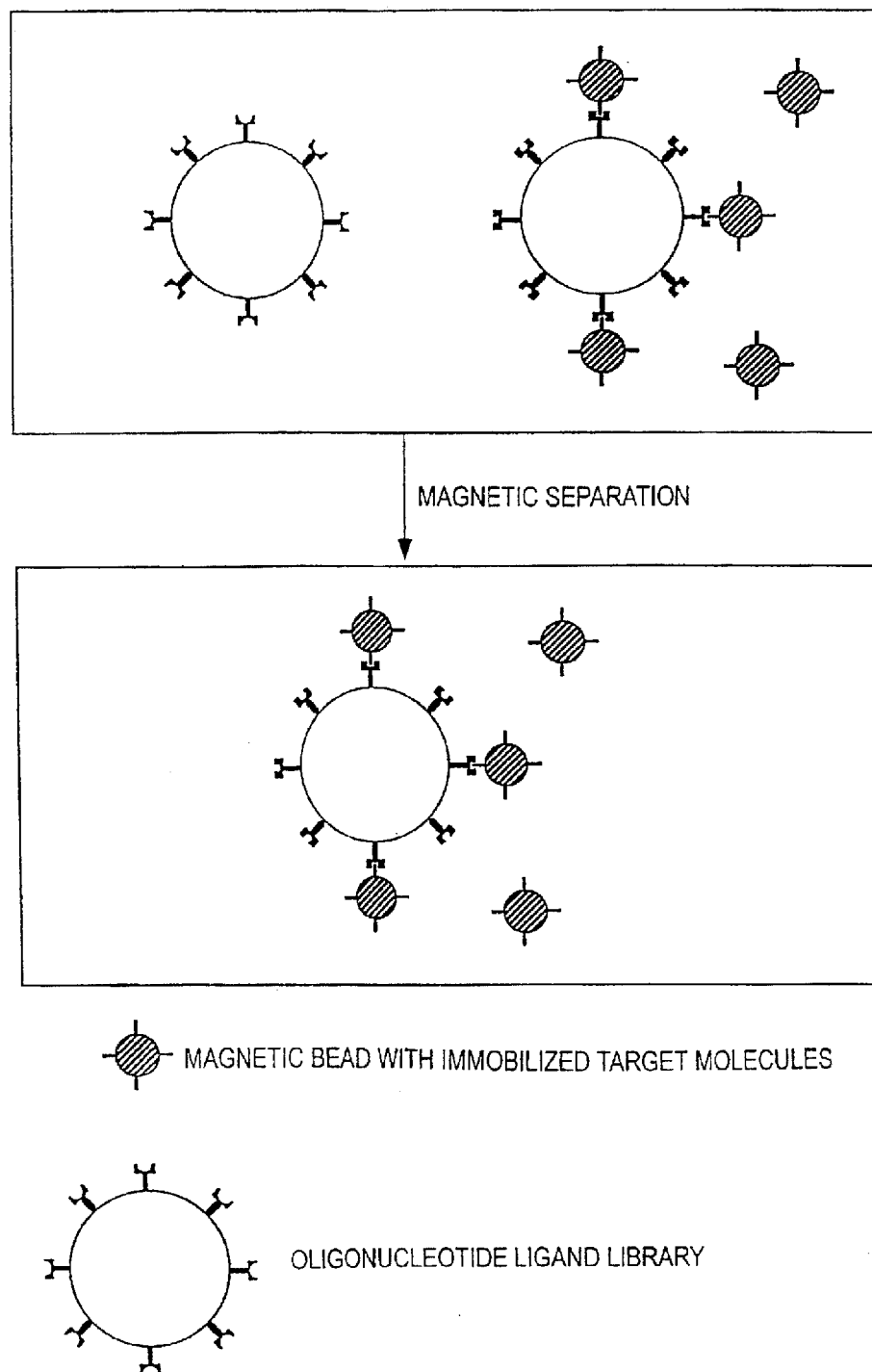


Fig. 1

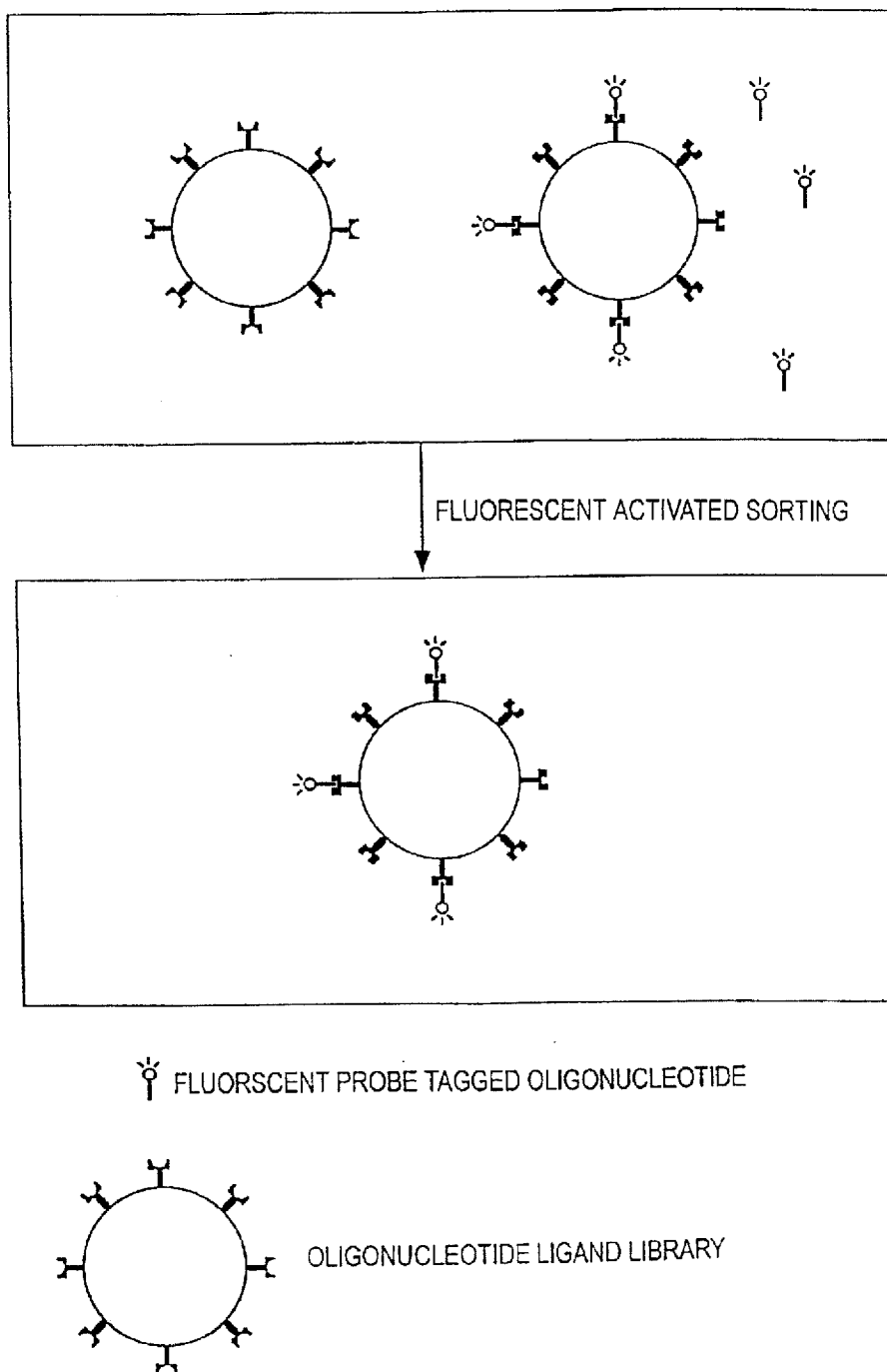


Fig. 2

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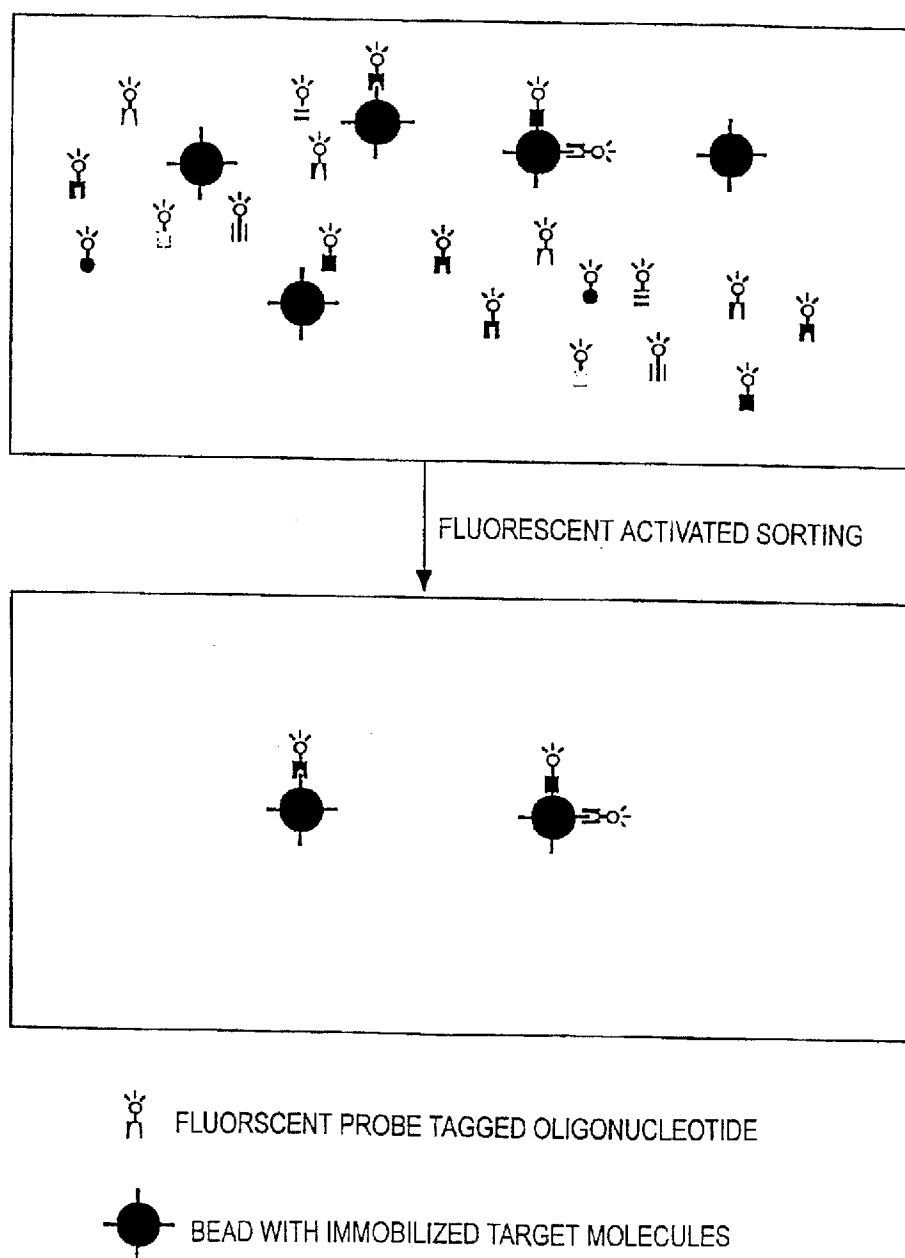


Fig. 3

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GROUP I

CONSENSUS TGTITGTT
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 F15 GGGGGCCAAGGATTCATGTTGTTGTTAACAGCTAGCGCCGCTT
 F21 GGGACCTCCAGAGCGAAGGTGTTCAACGTTGGTTAGGTTT
 F22 GGGACAAGGTTGTAGGCTTCAGTGTCTCTGTTGTTCCCTT
 F17 GCGATACGGAATATTGGTACCACCTATGTAGATGTTTGT

GROUP II

F8 GGGCCGGTGGTGAGATCAGAGTTAGTGAGTCTTCTGTCCGT
 F13 GGGAGCCGAATGTATGAAAGTGCTGAGTACAGGTGCTCGGGGGTT
 F19 GGGGCAGGGGGCGGACGAGCTAGTGCAGCATTTTGGTGGGT

Fig. 4

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FIG. 5.

1 2 3

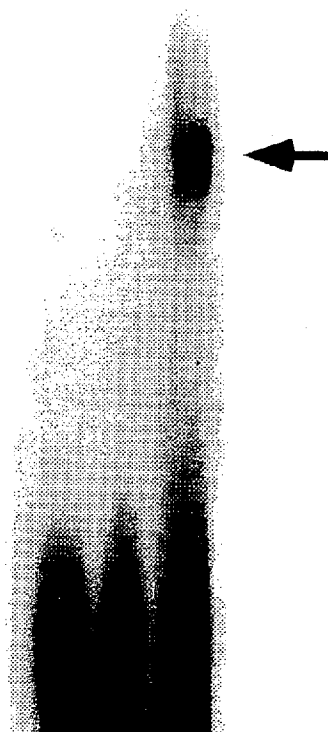
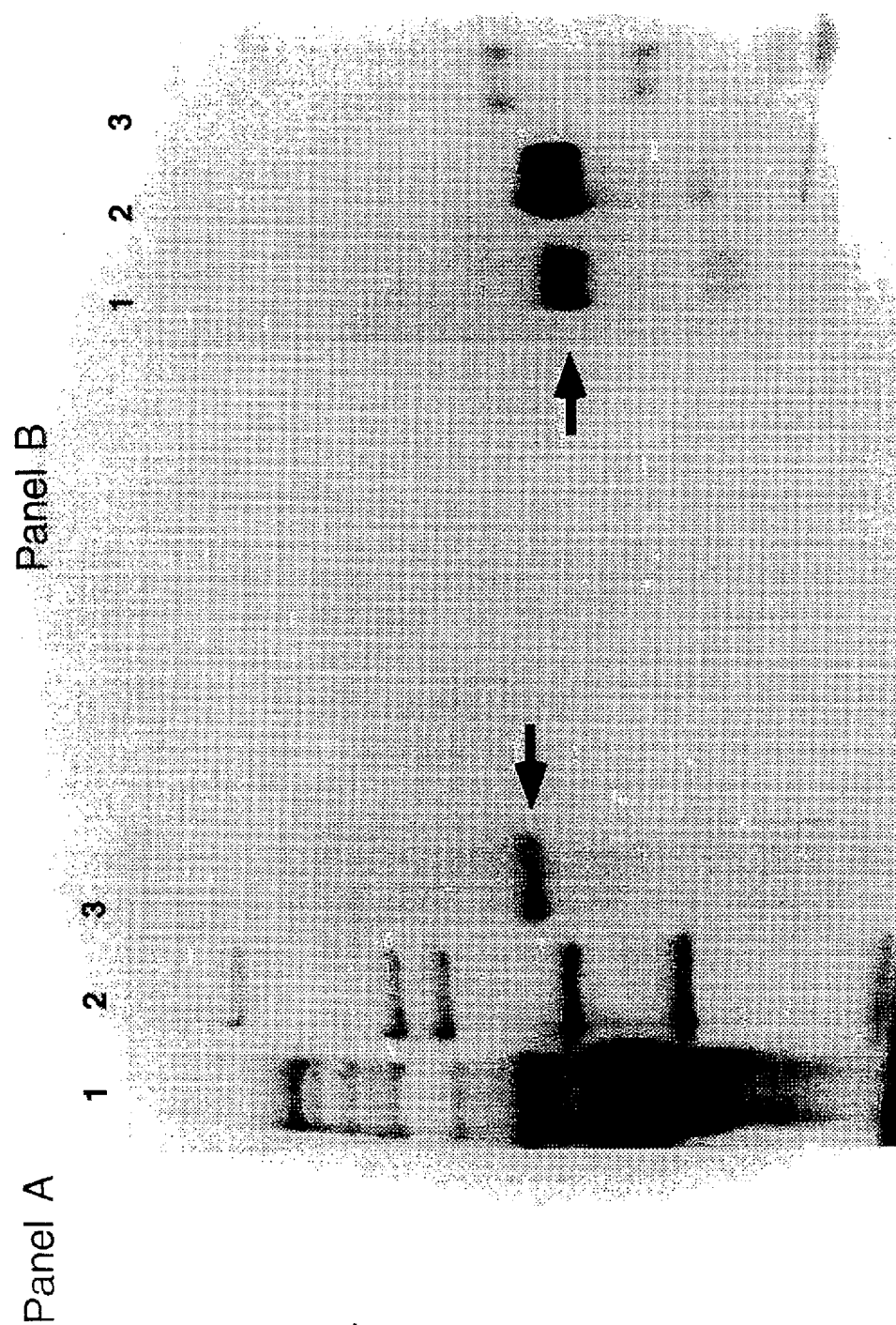


FIG. 6.



Sequence Listing Part

<110> BIOAGE PHARMACEUTICALS, INC.

<120> METHOD OF ISOLATING TARGET SPECIFIC OLIGONUCLEOTIDE LIGANDS

5 <130> Chen, Anthony C.
Registration No. 38,673
Docket Number: 229/191

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<151> April 20, 1998

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INTERNATIONAL SEARCH REPORT

International Application No.

PC/US 99/08561

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C1201/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C120

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 94 02515 A (BUNSEN RUSH LAB INC) 3 February 1994 (1994-02-03) * see especially page 8, paragraph 3 - page 9, paragraph 2, and claims 7 and 10 * the whole document ---	1,2,6-13
Y	HUANG S-C ET AL.: "Binding of biotinylated DNA to streptavidin-coated polystyrene latex" ANALYTICAL BIOCHEMISTRY, vol. 222, 1994, pages 441-449, XP002114508 * see especially Fig. 4 * the whole document --- -/--	1,2,6-13

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

7 September 1999

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20/09/1999

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INTERNATIONAL SEARCH REPORT

International Application No

PC./US 99/08561

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FRY G ET AL.: "A new approach to template purification for sequencing applications using paramagnetic particles" BIOTECHNIQUES, vol. 13, no. 1, 1992, pages 124-131, XP002114509 the whole document ---	1,2,6,7
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A	BOCK L C ET AL: "SELECTION OF SINGLE-STRANDED DNA MOLECULES THAT BIND AND INHIBIT HUMAN THROMBIN" NATURE, vol. 355, no. 6360, 6 February 1992 (1992-02-06), pages 564-566, XP000453533 ISSN: 0028-0836 cited in the application the whole document ---	
A	TUERK C ET AL: "SYSTEMATIC EVOLUTION OF LIGANDS BY EXPONENTIAL ENRICHMENT: RNA LIGANDS TO BACTERIOPHAGE T4 DNA POLYMERASE" SCIENCE, vol. 249, 3 August 1990 (1990-08-03), pages 505-510, XP000647748 ISSN: 0036-8075 the whole document ---	
A	WO 92 14843 A (GILEAD SCIENCES INC) 3 September 1992 (1992-09-03) cited in the application the whole document -----	

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International Application No

PC./US 99/08561

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